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TITLE: Local Inhibition of HSP90 to Prevent Intimal Hyperplasia after Balloon Injury

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14. ABSTRACT
Peripheral arterial disease (PAD) remains a major threat to life and limb and represents a disabling and potentially fatal condition in the aging military and veteran population. Dyslipidemia is an important mechanism in the pathogenesis of PAD and the development of restenosis secondary to intimal hyperplasia (IH) after balloon angioplasty. IH is a complex process that begins by platelet activation, platelets then bind to the area of vascular injury releasing thrombospondin-1 (TSP-1) and platelet derived growth factor (PDGF). These in turn cause vascular smooth muscle (VSMC) migration into the area of injury where they begin to proliferate and produce extracellular matrix like hyaluronic acid (HyA). All of these processes clearly contribute to IH by regulating the arterial response to injury. Heat shock protein 90 (HSP90) is a molecular chaperone binds many signaling proteins regulating their final maturation. HSP90 is ubiquitously expressed and is important for normal cell function. However, aberrant activation of HSP90 can result in increased cell migration and proliferation. Inhibition of HSP90 has been in examined in states of aberrant cell growth such as cancer. The quintessential HSP90 inhibitor is the natural product geldanamycin, however, geldanamycin exhibits a relatively high toxicity. Several derivatives of geldanamycin have been created that have significantly less toxicity and are in clinical trials for cancer therapy.

15. SUBJECT TERMS

None listed

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1. **INTRODUCTION:**

Peripheral arterial disease (PAD) is a significant health problem that affects the aging military and veteran population, ranging from lifestyle limiting claudication to major amputation and death. Further, there is growing rate of disorders in Cardiovascular Health, one of the FY15 PRMRP Topic Areas, in the military and veteran population leading to the development of PAD. Endovascular interventions are among the fastest growing treatments for PAD; however, restenosis secondary to intimal hyperplasia (IH) remains a major cause of treatment failure. The mechanisms of IH are still being elucidated and the optimal therapies to prevent IH in patients with PAD are unknown. Modes of delivering therapy, such as HSP90 inhibitors, targeting sites of injury are needed to maximize effectiveness against restenosis and occlusion. The information gained from these studies is expected to yield a detailed understanding of the role of HSP90 in IH, and discover the optimal HSP90 inhibitor delivery modality for preventing IH. *The key contribution of the proposed research will provide a major advance that is expected to lead to development of a new treatment strategy to prevent restenosis after angioplasty for PAD lesions.* Once such a strategy is available, the potential will exist for effective therapy to decrease the incidence of restenosis after balloon angioplasty. These studies will expand our understanding of the pathological cellular and molecular effects induced by vascular injury and PAD. The studies from this proposal will provide the knowledge needed to develop new methods of treatment that will ultimately improve healthcare for veterans with PAD. Further, these studies will identify key intracellular signaling intermediates that will be the subject of future applications.

2. **KEYWORDS:** Intimal Hyperplasia, HSP90, Heat Shock Protein 90, Smooth Muscle, MicroRNA
3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Specific Aim 1: Elucidate the effects of HSP90 inhibitors on VSMC migration and proliferation and expression of mir-17~92 cluster.	**Timeline**	**Percent Completed**
Major Task 1	Months	
Define the optimal 17-AAG and 17-DMAG. concentration to inhibit VSMC migration and proliferation. VSMCs are human aortic vascular smooth muscle cells from Cell Applications, San Diego, CA.	4	75%
Determine if 17-AAG and 17-DMAG inhibit or increase mir-17~92 cluster expression.	4	30%
Milestone(s) Achieved Will have a clear understanding of the effect of HSP90 VSMC migration, proliferation, mir-17~92 cluster expression and which is the more potent and least toxic inhibitor.	8	52.5%
Local IACUC Approval	3	100%
Milestone Achieved: HRPO/ACURO/IACUC Approval	6	100%
Specific Aim 2: Ascertain the optimal modality of HSP90 inhibitor delivery to reduce IH in an animal model of arterial balloon injury.		
Major Task 2		
Perform arterial injury and treatment to 36 Sprague-Dawley rats	6	100%

Histochemical analysis of the carotid artery sections. Hematoxylin and Eosin staining	3	0%
Milestone(s) Achieved: Will determine if HSP90 inhibition reduces intimal hyperplasia and which delivery modality is optimal.	12	50%

- **What was accomplished under these goals?**
- 1) Major activities: Preparing IACUC documents. Cell culture and viability testing, optimizing proliferation and migration assays, perfecting microRNA extraction, perfecting cDNA creation, optimizing MicroRNA qrtPCR, carotid artery balloon injury in the rat (36 animals) with DMAG treatment using different delivery modalities (pluronic gel, intraluminal or both). Harvest and fixation of tissues 14 days post injury.

  2) Specific objectives: Obtain IACUC approval. Elucidate the effects of HSP90 inhibitors on VSMC migration and proliferation and expression of mir-17~92 cluster. Determine the optimal concentration of HSP90 inhibitors to arrest VSMC function. Ascertain the optimal modality of HSP90 inhibitor delivery to reduce IH in an animal model of arterial balloon injury.

  3) Key outcomes: The optimal concentration of DMAG and AAG was found to be in the nanomolar range. Inhibition of HSP90 reduced proliferation to PDGF (Figure 1), Fibronectin (Figure 2) and Fetal Bovine Serum (FBS, Figure 3).

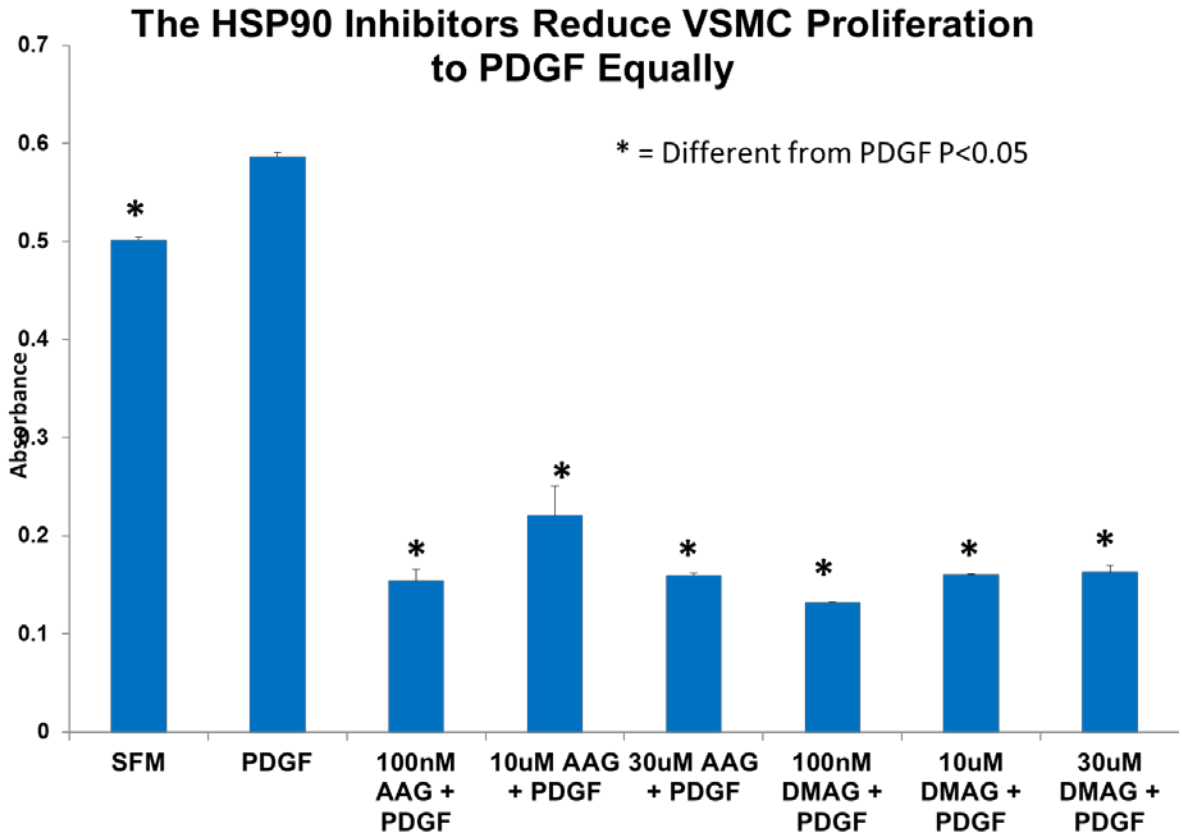

Figure 1.

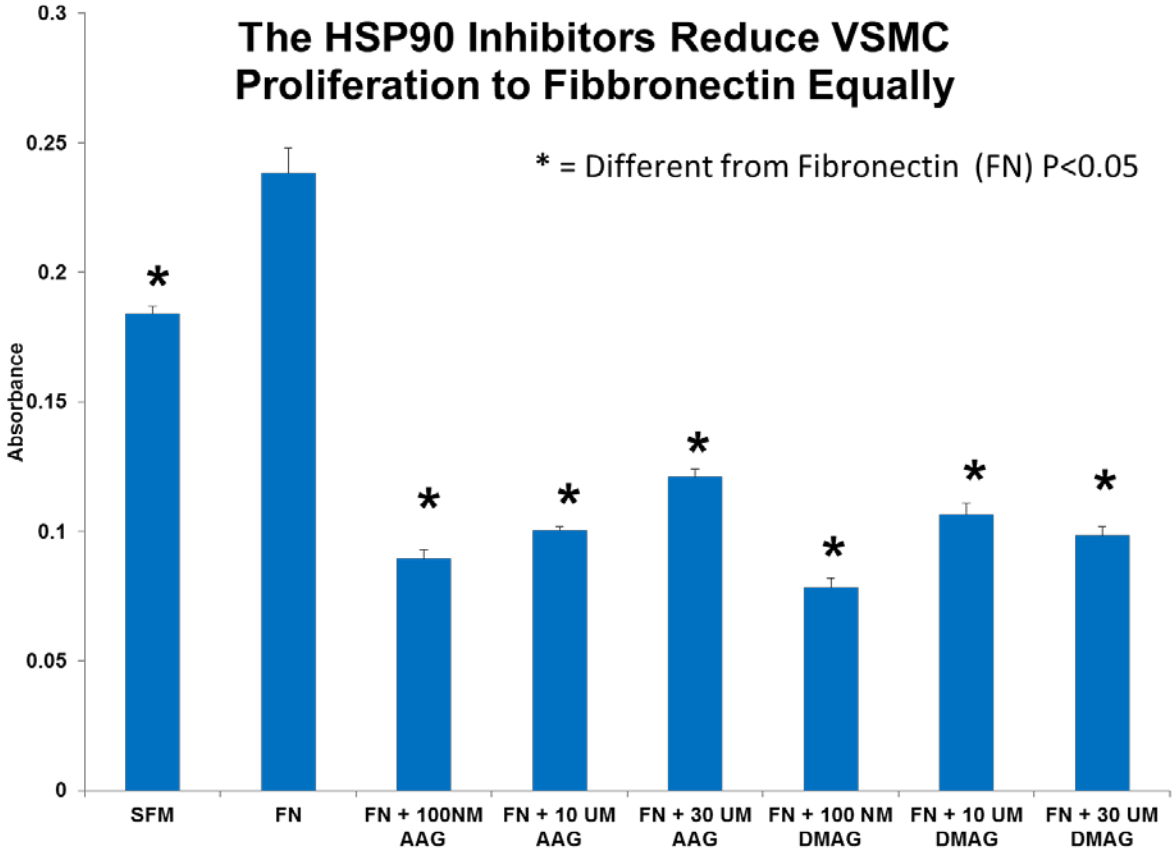

Figure 2.

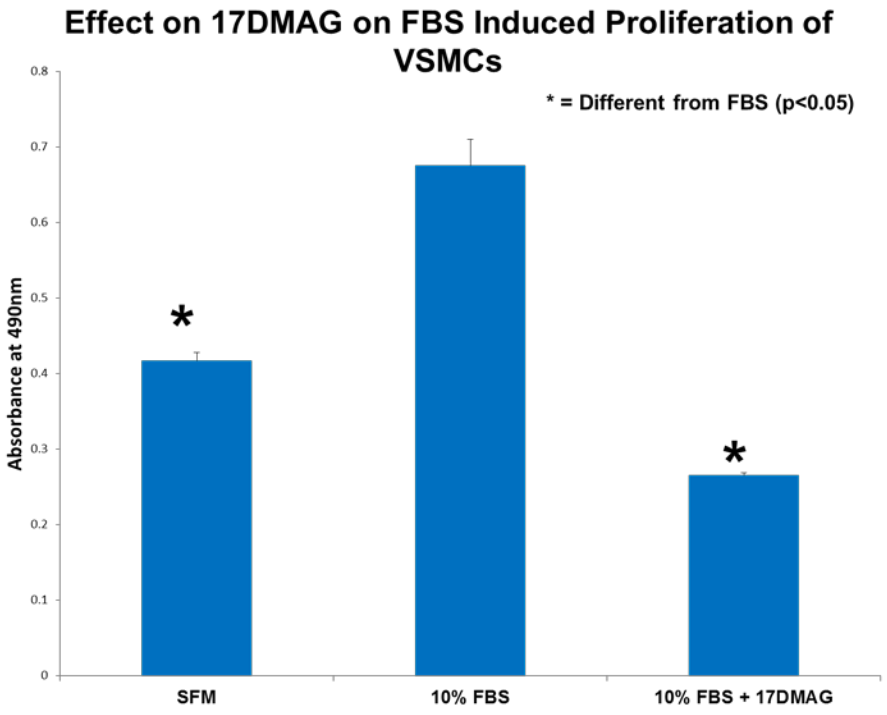

Figure 3.

Further there was no difference in the magnitude of the effect of DMAG and AAG on vascular smooth muscle cells (VSMCs) at any dose, so it was decided to use the water soluble DMAG for the in vivo work at 500 nM.

Stated goals not met: The effect of HSP90 inhibitors on VSMC migration and the expression of the mir-17~92 cluster in VSMCS have not been fully completed but it is expected that these experiments will be finished by the end of 2017. As noted earlier the animal surgeries are complete and the tissue of all groups has been harvested. We hope that the new histology lab we are working with con begin processing the samples in November of 2017 and that the analysis will be completed by January or February 2018. At that point all data will be integrated and we will begin writing the manuscript.

Methods

(1) Cell Culture: Human VSMCs were obtained from Cell Applications (San Diego, CA). All VSMCs were maintained in Smooth muscle cell Growth Media (Cell applications. San Diego, CA) VSMCs were confirmed by their typical morphology and hill-and-valley pattern. Passages 2-5 were used.

(2) Proliferation Assay: Cell proliferation was assessed in growth-arrested VSMCs grown in a 96 well plate with 5000 cells/well. Cell were treated as described in the experimental design and proliferation assessed with a commercially available colorimetric assay (Cell Titer 96 Aqueous Cell Proliferation Assay, Promega, Madison, WI).

(3) Cell Viability: Cell viability was determined with the Evan's Blue exclusion assay using the Countess Cell counter.

(4) qrtPCR: cDNA was prepared using the High Capacity cDNA Revers Transcription kit (Applied Biosystems. Grand Island, NY). qPCR was performed using the Quantstudio 7 (Applied Biosystems. Grand Island, NY). Reactions were 20 µl and products amplified for 40 cycles.

(5) Carotid Artery Balloon Injury: Sprague-Dawley rats (male, 10-12 wks. old, Harlan Laboratories, Indianapolis, IN) were randomized to treatment or control groups. Animals werehoused in an AALAC accredited Laboratory Animal facility, housed in filter top cages (4 per cage) with ad libitum access to food and water. Animals were monitored daily for changes in health. Rats were anesthetized (isoflurane, least stressful route of anesthesia) in the morning in a rodent surgical laboratory. Via carotid artery cutdown, the left CCA and its bifurcation was exposed. An angioplasty catheter (2mm x 6mm, Sprinter, Medtronic, Minneapolis, MN) was placed into the CCA via the external carotid artery, the balloon inflated (5 atmospheres, 5 min). Animals were euthanized and specimens processed (bilateral CCAs perfusion fixed, and harvested) For topical therapy, HSP90 inhibitor will be suspended in 20% pluronic gel (F-127, Anaspec, Fremont, CA). For the intraluminal therapy, after the balloon injury, PE tubing Atrium, Hudson, NH) was inserted and the HSP90 inhibitor in saline will be infused into the clamped vessel at a pressure of 2 atmospheres for 5 minutes.

(6) Statistical Analysis: *In vitro* experiments were done in triplicate using separate cell lines. Data was tested by ANOVA with post hoc testing or Student's t-test when appropriate. *p*-values <0.05 was considered to be significant.

- **What opportunities for training and professional development has the project provided?** Nothing to Report
- **How were the results disseminated to communities of interest?** Nothing to Report
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - During the next reporting period we plan to analyze the animal tissues, finish the migration and microRNA experiments. Depending on the data being positive we would plan to present the results at a national meeting and publish the manuscript.

4. **IMPACT:***:*
- **What was the impact on the development of the principal discipline(s) of the project?** Nothing to Report
- **What was the impact on other disciplines?** Nothing to Report
- **What was the impact on technology transfer?** Nothing to Report
- **What was the impact on society beyond science and technology?** Nothing to Report

5. **CHANGES/PROBLEMS:**
- **Changes in approach and reasons for change.** Nothing to Report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - We have had some difficulty in optimizing the protocol for creating cDNA from MicroRNA. We have tried three methods and now feel comfortable with the improved protocol. There has been a delay in processing the animal slides due to the pathology laboratory being understaffed and overwhelmed. We have located a different lab at Cornell University and are setting up the protocols to process the tissues for analysis. The PI had to have knee surgery in January 2017 followed by a month of rehabilitation so there was a delay due to health. Further the Post-doctoral fellow did not start until July 2017 which has delayed the *in vitro* work.
- **Changes that had a significant impact on expenditures.** Nothing to Report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents** Nothing to Report
- **Significant changes in use or care of human subjects** Nothing to Report
- **Significant changes in use or care of vertebrate animals.** Nothing to Report
- **Significant changes in use of biohazards and/or select agents** Nothing to Report

6. **PRODUCTS:**
- **Publications, conference papers, and presentations** Nothing to Report
  Journal publications. Nothing to Report
  - **Books or other non-periodical, one-time publications.** Nothing to Report
  - **Other publications, conference papers, and presentations.** Nothing to Report
- **Website(s) or other Internet site(s)** Nothing to Report
  Technologies or techniques Nothing to Report

7. **Inventions, patent applications, and/or licenses** Nothing to Report
   Other Products Nothing to Report
   PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
- **What individuals have worked on the project?**

Name:	*Kristopher Maier*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-0987-4751
Nearest person month worked:	*no change*
Contribution to Project:	*no change*
Funding Support:	*NIH R01*

Name:	*Vivian Gahtan*
Project Role:	*Co-Investigator*
Nearest person month worked:	*no change*
Contribution to Project:	*no change*
Funding Support:	*NIH R01*
Name:	*David Bruch*
Project Role:	*Senior Research Technologist*
Nearest person month worked:	*no change*
Contribution to Project:	*no change*
Funding Support:	*no change*
Name:	*Furqan Muqri, MD*
Project Role:	*Post-Doctoral Fellow*
Nearest person month worked:	*3*
Contribution to Project:	*Laboratory assays*
Funding Support:	*no change*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Drs. Maier and Gahtan received a multi-PI NIH R01 grant entitled "The Role of the Thrombospondins in Intimal Hyperplasia. There is no scientific or effort overlap with this grant and the current one in this report.
- **What other organizations were involved as partners?** Nothing to Report